Evidence for epidermal growth factor receptor as negative-feedback control in aldosterone-induced Na\textsuperscript{+} reabsorption

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Grossmann, Claudia, Ruth Freudinger, Sigrid Mildenberger, Alexander W. Krug, and Michael Gekle. Evidence for epidermal growth factor receptor as negative-feedback control in aldosterone-induced Na\textsuperscript{+} reabsorption. Am J Physiol Renal Physiol 286: F1226–F1231, 2004. First published January 28, 2004; 10.1152/ajprenal.00378.2003.—Aldosterone enhances Na\textsuperscript{+} reabsorption via epithelial Na\textsuperscript{+} channels (ENaC). Aldosterone also stimulates the protein kinase ERK1/2- and the epidermal growth factor (EGF) receptor (EGFR)-signaling pathway. Yet EGF and ERK1/2 are known inhibitors of ENaC-mediated Na\textsuperscript{+} reabsorption. In the present study, using the well-established Madin-Darby canine kidney C7 cell line, we tested the hypothesis that EGF represents a negative-feedback control for chronic aldosterone-induced Na\textsuperscript{+} reabsorption [amiloride-inhibitable short-circuit current (I\textsubscript{sc})]. Mineralocorticoid receptor expression was confirmed by RT-PCR and Western blot analysis. Aldosterone enhanced ERK1/2 phosphorylation in an EGFR-dependent way. Furthermore, aldosterone stimulated EGFR expression. Aldosterone (10 nmol/l) induced a small transient increase in I\textsubscript{sc}, indicating constitutive ENaC inhibition. Aldosterone exerted a significantly larger effect in the presence of U-0126 than without U-0126. EGF (10 \textmu mol/l) inhibited I\textsubscript{sc}, whereas inhibition of EGFR kinase by tyrphostin AG-1478 (100 nmol/l) enhanced I\textsubscript{sc}. Aldosterone was more effective in the presence of AG-1478 than without AG-1478. In summary, we propose that the EGF-signaling cascade can serve as a negative-feedback control to limit the effect of aldosterone-induced Na\textsuperscript{+} reabsorption.

extracellular signal-regulated kinase 1/2; Madin-Darby canine kidney C7 cells

SINCE THE DISCOVERY THAT aldosterone regulates NaCl reabsorption in epithelia, efforts have been expended in delineating the mechanisms of its action as well as its interaction with other hormones relevant for Na\textsuperscript{+} transport (27). Aldosterone binds to the cytosolic mineralocorticoid receptor (MR) (1), which modulates expression of such proteins as the epithelial Na\textsuperscript{+} channel (ENaC), the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, or the serum- and glucocorticoid-regulated kinase, thereby affecting Na\textsuperscript{+} reabsorption (1, 5, 11, 32, 35, 36). Aldosterone has the ability to interact with peptide hormone signaling (22), similar to angiotensin II and vasopressin (29, 39). Another important peptide-signaling “system” with respect to aldosterone is the epidermal growth factor (EGF) and its receptor (EGFR) (13, 22). It has been shown that aldosterone stimulates the EGF-EGFR-ERK1/2-signaling module (12, 22, 33). Aldosterone can also stimulate EGFR expression (21, 22). EGF regulates cell proliferation, differentiation, and tissue repair (18, 24). Furthermore, EGF affects epithelial salt transport in a cell-specific manner, leading to enhanced or reduced salt reabsorption (10, 19, 20, 26, 38). In renal collecting duct cells, EGF inhibits Na\textsuperscript{+} reabsorption via ERK1/2 (30). Thus aldosterone stimulates a signaling pathway (EGF-EGFR-ERK1/2) that acts in the opposite direction with respect to Na\textsuperscript{+} reabsorption in collecting duct cells. The potential functional consequence of this interaction is not known. Activation of an antagonistic signaling system would impose a negative feedback on the action of aldosterone. In the present study, we tested the hypothesis that EGF can act as a negative-feedback control for chronic aldosterone-induced Na\textsuperscript{+} reabsorption in a cell culture system. For this purpose, we used the Madin-Darby canine kidney (MDCK) cell clone C7, which shows characteristics of principal cells, including ENaC-mediated Na\textsuperscript{+} transport (3, 15, 16, 23, 28). The data suggest that the EGF-signaling cascade can serve as a negative-feedback control to limit the effect of aldosterone-induced Na\textsuperscript{+} reabsorption, at least in MDCK C7 cells.

METHODS

Cell culture. We used a subtype of MDCK cells, denominated C7 (MDCK C7), which has been cloned recently in our laboratory (16). MDCK C7 cells show characteristics of principal cells, including amiloride-sensitive Na\textsuperscript{+} transport and aldosterone responsiveness (3, 15, 16, 23, 28). Cells were cultivated in MEM supplemented with 10% fetal calf serum at 37°C and 5% CO\textsubscript{2}. At 24 h before the experiments, serum was removed from the medium. The cells were cultivated on permeable supports (Becton Dickinson, Heidelberg, Germany) or in 96-well plates [for phosphorylated ERK1/2 (pERK1/2) ELISA]. To rule out toxic effects during treatment, lactate dehydrogenase release was measured (2). No significant increase in lactate dehydrogenase release was detected.

Transmonolayer measurements. For the investigation of short-circuit current (I\textsubscript{sc}) and transepithelial resistance (R\textsubscript{te}), cells were seeded near density [5 × 10\textsuperscript{5} cells/cm\textsuperscript{2}] on 4.9-cm\textsuperscript{2} permeable filters (Falcon, Heidelberg, Germany) that were placed in six-well dishes. Because R\textsubscript{te} and transepithelial potential difference (PD\textsubscript{te}) were measured repeatedly over a period of several days, we used the voltmeter system (EVOM, WPI, Sarasota, FL). The measurements were performed immediately after removal of the filters from the incubator, preventing a significant alteration of the milieu. Temperature was 32–36°C. From previous pH measurements, we know that CO\textsubscript{2} remains stable during this time period. C7 cells lower pH slightly to ~7.3. Resistance of the filters alone (120 ± 5 \textmu Omega cm\textsuperscript{2}, n =
20) was measured in parallel and subtracted from the values measured for the monolayers. The equivalent \( I_{sc} \) was calculated as \( P_{D_2}/R_{te} \) according to Ohm’s law. Control experiments showed no loss of medium due to evaporation during the experimental period.

A second approach to investigate transepithelial reabsorptive transport was determination of dome formation (25, 34). Cells were seeded in 24-well dishes with a marked area of 25 mm² each, and the number of \( \geq 50\mu m \) diameter domes in the marked areas was counted.

Western blot analysis. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold Trition X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 200 \( \mu \)M sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 \( \mu \)g/ml leupeptin, 1 \( \mu \)g pepstatin A, 40 mg/l bestatin, 2 \( mg/l \) aprotinin, and 1% Triton X-100) or RIPA buffer for 25 min at 4°C. Insoluble material was removed by centrifugation at 12,000 \( g \) for 15 min at 4°C. Cell lysates were matched for protein content, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Subsequently, membranes were blotted with a rabbit anti-pERK1/2 antibody (1:1,000; New England Biolabs, Beverly, MA), anti-EGFR antibody (1:1,000; catalog no. sc-03, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-MR antibody (1:1,000; catalog no. sc-6860, Santa Cruz Biotechnology). The bound primary antibody was visualized using horseradish peroxidase-conjugated secondary IgG (1:25,000) and the ECL system (Amersham). Linearity of the signal has been verified by serial dilution as recommended by the manufacturer. Densitometric analysis was performed using Signagel 1.05 software (Jandel, Corte Madera, CA). We routinely used the flood, as well as the line, method to determine density. For the line method, three independent lines were quantified per band. Both methods gave similar results.

Quantification of ERK1/2 phosphorylation by ELISA. For quantification of ERK1/2 phosphorylation, we performed pERK1/2 ELISA according to Versteeg et al. (37). In control experiments, we compared the effect of EGF determined by Western blot analysis and pERK1/2 ELISA and found no significant difference, as described elsewhere (13). Thus the results obtained by Western blot analysis and pERK1/2 ELISA were pooled. The cells were seeded in 96-well plates (Maxisorp, Nunc) and serum starved for 24 h before the experiment. After stimulation, the cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature and washed three times with PBS containing 0.1% Triton X-100. Endogenous peroxidase was quenched with 0.6% H₂O₂ in PBS-Triton X-100 for 20 min. The cells were washed three times in PBS-Triton X-100, blocked with 10% fetal calf serum in PBS-Triton X-100 for 1 h, and incubated overnight with the above-described primary antibody (1:1,000) in PBS-Triton X-100 containing 5% BSA at 4°C. On the following day, the cells were washed three times with PBS-Triton X-100 for 5 min and incubated with the secondary antibody (peroxidase-conjugated mouse anti-rabbit antibody, diluted 1:10,000) in PBS-Triton X-100 containing 5% BSA at 4°C. For the multiwell multilabel counter (Victor 2, Wallac, Turku, Finland). After the peroxidase reaction, the cells were washed twice with PBS-Triton X-100 and twice with demineralized water. After the wells were dried for 5 min, trypan blue solution (100 \( \mu l \), 0.2% in PBS) was added for 5 min at room temperature. Subsequently, the cells were washed four times with demineralized water, 1% SDS solution (100 \( \mu l \)) was added, and the cells were incubated on a shaker for 1 h at room temperature. Finally, the absorbance was measured at 595 nm with the ELISA reader (see above).

RT-PCR. RNA was isolated using the TRIzol reagent (Life Technologies). RT-PCR was performed using the SuperScript One-Step RT-PCR kit (Invitrogen, Karlsruhe, Germany). The MR primers were as follows: 5’ATCACGATCCGGCTAGACC (forward) and 5’CCCATATTGCCATCTGGAAG (reverse). The reaction was carried out at an annealing temperature of 55°C for 35 cycles; product size was 244 bp. The primers span intron B of the MR. Therefore, the expected product will be obtained only if the template is mRNA.

Materials. U-0126, tyrphostin AG-1478, and compound 56 were obtained from Calbiochem (Bad Soden, Germany). Unless stated otherwise, all other materials were obtained from Sigma (Munich, Germany).

Statistics. Values are means ± SE; \( n \) represents the number of tissue culture dishes investigated. Significance of difference was tested by Student’s t-test or ANOVA as applicable. Differences were considered significant if \( P < 0.05 \). Cells from at least two different passages were used for each experimental series.

RESULTS AND DISCUSSION

Because we wanted to investigate the long-term effects of aldosterone on Na⁺ transport, first, we tested whether the parameters of C7 cells cultivated on permeable supports are stable. Figure 1A shows that \( R_{te} \) and \( I_{sc} \) are stable over 72 h once the monolayers are confluent. The transepithelial potential was also constant (5.2 ± 0.4 mV apical compartment negative, \( n = 24 \)). ENaC-mediated Na⁺ transport in C7 cells...
has been shown in several studies (3, 23). The data shown in Fig. 1B confirm these studies. *I* _sc_ is almost completely inhibited by 10 μmol/l amiloride. Similar results were obtained for all time periods and also during stimulation of *I* _sc_. Thus *I* _sc_ is a suitable functional parameter for estimation of ENaC function. Although MDCK cells have been widely used to study effects of mineralocorticoids, expression of the MR in these cells has not been definitively determined. Western blot analysis showed a band of the expected size (Fig. 1C) in MDCK C7 cells, but not in opossum kidney cells, used as a negative control. MDCK C11 cells, for which we previously demonstrated expression of the MR by RT-PCR, also gave a band of the expected size (21). In addition, using an MR primer pair that spans intron B of the MR, we performed RT-PCR with RNA from MDCK C7 cells. The expected product obtained from RNA has a size of 244 bp. As shown in Fig. 1D, we obtained a product of the expected size. Thus MDCK C7 cells used in this study express endogenous MR spontaneously and represent a model system to investigate the modulation of ENaC function by mineralocorticoids.

Because we wanted to test the hypothesis that upregulation of the EGFR-signaling pathway by aldosterone may serve as a negative-feedback control system, we had to test whether aldosterone also exerts this effect in C7 cells. Because MDCK cells express EGF in addition to EGFR, they are subject to an autocrine activation loop, which is responsible for basal ERK1/2 phosphorylation (13, 31). We tested whether this autocrine activation loop is also present in our C7 clone. Using the ELISA technique described by Versteeg et al. (37) and adapted by us (22), we determined the effect of the EGFR kinase inhibitor tyrphostin AG-1478 on basal and stimulated ERK1/2 phosphorylation in C7 cells. EGF (10 μg/l) stimulated ERK1/2 phosphorylation to 210 ± 15% of control after 5 min (*n* = 9). Tyrphostin AG-1478 (100 nmol/l) reduced basal ERK1/2 phosphorylation almost completely (to 15 ± 6% of control, *n* = 6) and prevented the effect of EGF (22 ± 7% of control, *n* = 6). These data show that an autocrine activation loop involving EGF and EGFR is active in C7 cells also.

Figure 2A shows that long-term aldosterone exposure leads to enhanced ERK1/2 phosphorylation with only a minor increase in total ERK1/2 expression. Exposure to EGF also enhances ERK1/2 phosphorylation (Fig. 2A). Thus, in C7 cells, aldosterone and EGF elicit prolonged ERK1/2 activation (4). Using a pharmacological approach, we tested whether aldoste-
Aldosterone alone elicits a significant increase in pERK1/2. To test whether aldosterone stimulates EGFR expression, as observed elsewhere in other cell types and after heterologous MR expression (17, 21). The Western blot analysis in Fig. 2C shows that aldosterone exposure stimulates EGFR expression in C7 cells after 24, 48, and 72 h. Expression was increased to 180% after 24 h, to 160% after 48 h, and to 190% after 72 h (average of 2 or 3 blots). In an additional series of experiments, we determined aldosterone-induced ERK phosphorylation after 24, 48, and 72 h and tested the inhibitory effect of two EGFR kinase inhibitors: tyrphostin AG-1478 (100 nmol/l) and compound 56 (100 nmol/l) (7). As shown in Fig. 2D, both inhibitors prevented aldosterone-induced ERK phosphorylation. Taken together, these data support the hypothesis that aldosterone stimulates the EGF-EGFR-signaling cascade in C7 cells. The question now was whether this signaling cascade antagonizes the action of aldosterone.

We and others previously showed that aldosterone, at high concentrations (1 μmol/l), stimulates \(I_\text{sc} \) in C7 cells (3, 14, 16, 23). In this concentration range, aldosterone can act via the MR as well as via the glucocorticoid receptor. Therefore, we tested the effect of 10 nmol/l aldosterone on \(I_\text{sc}\). Figure 3A shows that aldosterone alone elicits a significant, although small, increase in \(I_\text{sc}\) only after 24 h. Later, \(I_\text{sc}\) returned to control level. One explanation for the transient effect of aldosterone is the existence of a negative-feedback loop. To test whether the inhibitory action of ERK1/2 contributes to a putative negative-feedback loop and limits the effect of aldosterone, we added an inhibitor of ERK1/2 activation, U-0126 (10 μmol/l). U-0126 alone led to a significant increase in \(I_\text{sc}\) (Fig. 3A). These data are in good agreement with other studies describing the inhibitory action of ERK1/2 on ENaC (6). Furthermore, these data support our hypothesis of tonic suppression of ENaC by the EGF-EGFR autocrine activation loop. Interestingly, aldosterone stimulated \(I_\text{sc}\) significantly in the presence of U-0126 (Fig. 3A). These data are in good agreement with the model depicted in Fig. 3B indicating that ERK1/2 antagonizes the stimulatory action of aldosterone on Na\(^+\) transport.

A classic stimulator of ERK1/2 is EGF, which acts via the EGFR. Because MDCK cells express EGFR (13), also shown in Fig. 2C, we tested whether it is possible to reproduce the inhibitory effect of EGF on ENaC (30) in C7 cells. Application of EGF (10 μg/l) reduced \(I_\text{sc}\) significantly (Fig. 4A). The effect of EGF was strongest after 24 h, with a tendency to decline thereafter. The reason for this partial transient action of EGF is not known. Possibly, the concentration of EGF in the medium decreased or the cells reacted with an adaptive response. These data show that EGF also inhibits ENaC in C7 cells.

To test whether the autocrine EGF-EGFR activation loop is involved in the inhibition of aldosterone-stimulated ENaC, we tested the effect of tyrphostin AG-1478 on \(I_\text{sc}\). Application of AG-1478 led to a slight increase in \(I_\text{sc}\), similar to U-0126 (Fig. 4B). Furthermore, AG-1478 and U-0126 prevented the action of EGF. In the presence of one of the two substances, EGF had no significant effect. These data support the hypothesis of an autocrine EGF-EGFR activation loop suppressing ENaC. In the presence of AG-1478, aldosterone significantly stimulated \(I_\text{sc}\) (Fig. 4B), and the stimulatory effect was no longer transient (cf. Fig. 3A), indicating the absence of the negative-feedback control consisting of the autocrine EGF-EGFR activation loop. Taken together, these data allow us to extend our model (Fig. 4C): EGF antagonizes the stimulatory action of aldosterone on Na\(^+\) transport via ERK1/2.

In a final series of experiments, we investigated dome formation, another measure for reabsorptive transport (25, 34).
Domes are localized regions of the monolayer sheet that are lifted off the culture dish as the result of net apical-to-basolateral movement of osmotically active constituents and water. Aldosterone induced a slight increase in dome formation (Fig. 5) that was significantly potentiated by 100 nmol/l AG-1478. Dome formation was inhibited by amiloride with an EC50 of 1.26 μmol/l. Thus dome formation results mainly from amiloride-sensitive Na reabsorption. In some preliminary experiments, we overexpressed human EGFR (HER1) in C7 cells (22) and tested whether the effect of aldosterone is reduced. In mock-transfected cells, 10 nmol/l aldosterone induced a slight increase in Na+ transport (+25 ± 10%, n = 6); this was not the case in HER1-transfected cells (+2 ± 9%, n = 6). These data support the hypothesis that EGFR acts as a negative feedback on aldosterone.

From our data, we conclude that EGF inhibits ENaC in C7 cells, as shown for the mCT1 cell line (30). Furthermore, the EGF-signaling pathway can exert a tonic suppressive effect on the action of aldosterone with respect to ENaC. Finally, aldosterone exerts a long-term stimulatory action on the EGF-
signaling pathway. Thus aldosterone upregulates a signaling system that antagonizes its own action. Inhibition of the EGF-signaling pathway enhances the action of aldosterone. All these components form a negative-feedback loop and support the hypothesis that the EGF-signaling pathway helps prevent over-stimulation of Na\(^+\) reabsorption by aldosterone. This is still a working hypothesis, and further work is required to show that these mechanisms are also active in native tissue and affect Na\(^+\) excretion in vivo.

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